Remarks

Claims 1-4, 6-9, 11, 12, 14, 15, 18-21, 23-31, 43-50, and 52-68 were pending in the subject application. By this Amendment, claims 1, 3, 6-8, 15, 18, 43, 45-48, 50, 53-58, 60, 62, 64, and 68 have been amended, claims 2, 4, 14, 20, 21, 23-31, 44, 59, 61, 63, 65, and 67 have been cancelled, and new claims 69-73 have been added. The undersigned avers that no new matter is introduced by this amendment. Entry and consideration of the amendments presented herein is respectfully requested. It should be understood that the amendments presented herein have been made solely to expedite prosecution of the subject application to completion and should not be construed as an indication of the applicants' agreement with or acquiescence in the Examiner's position. Accordingly, claims 1, 3, 6-9, 11, 12, 15, 18, 19, 43, 45-50, 52-58, 60, 62, 64, 66, and 68-73 are currently before the Examiner for consideration. Favorable consideration of the pending claims is respectfully requested.

The applicants and the applicants' representative wish to thank Examiners Noble and Paras for the courtesy of the telephonic interview conducted with the undersigned and Mr. Jay Pattumudi on June 12, 2007, regarding the Office Action. The remarks and amendments set forth herein are consistent with the substance of the interview and are believed to address the outstanding issues as discussed during the interview.

Submitted herewith is a supplemental Information Disclosure Statement (IDS), accompanied by the form PTO/SB/08 and copies of the references listed therein. The applicants respectfully request that the references listed on the form PTO/SB/08 be considered and made of record in the subject application.

As an initial matter, the applicants note that the supplemental Information Disclosure Statement (IDS) submitted on February 21, 2007 was not acknowledged in the instant Office Action. The applicants reviewed the status of the subject application on the U.S. Patent Office's Patent Application Information Retrieval (PAIR) system and verified that the Patent Office has received the supplemental IDS. The applicants respectfully request that the Examiner consider the references listed on the Form PTO/SB/08 and make their consideration of record in the subject application.

By this amendment, the applicants have amended the specification at page 16, lines 21-32 through page 17, lines 1-16 to include the correct accession number and sequences of human IFN-γ. Recitation of Accession No. NM_000639 (and the nucleic acid sequence associated therewith) represent an obvious typographical error. Accession No. NM_00619 provides the nucleotide sequence for the human IFN-γ, as would be appreciated by one of ordinary skill in the art. The undersigned avers that no new matter is introduced by this amendment.

Claims 20, 21, 23-31, 54-58, 67, and 68 have been rejected under 35 U.S.C. §103(a) as being obvious over Hogan *et al.* (*Eur. J. Immunol.*, 1998, 28:413-423), in view of Li *et al.* (*J. Immunol.*, 1996, 157:3216-3219), Dow *et al.* (U.S. Patent No. 6,693,086), and O'Donnell *et al.* (*J. Immunol.*, 1999, 163:4246-4252). The applicants respectfully submit that the claimed invention is not obvious in view of the cited references.

When the prior art teaches away from combining certain known elements, discovery of a successful means of combining them is more likely to be non-obvious. *KSR International Co. v. Teleflex Inc.*, 550 U.S. _ 2007, citing *United States v. Adams*, 383 U.S. 39, 51-52 (1966). The combination of familiar elements according to known methods is likely to be obvious when it does no more than yield predictable results. *Id.* In this case, the references would not be combined by a person of ordinary skill in the art, where the references teach away from each other.

For example, the Hogan *et al.* publication (see page 418-419, last paragraph to beginning of page 419) states "although the protective effects of IL-12 were apparently mediated via the activity of endogenous IFN-gamma, in our study, the use of the former may nevertheless represent a superior approach to vector-directed gene therapy of allergy. We have found that gene transfer of IFN-gamma was far less protective against disease in this model (unpublished data) possibly because of the short half-life of the factor...." Hence, a person of ordinary skill in the art would not combine Hogan *et al.* with any of the cited references where the references teach away from each other. In addition, the applicants reiterate the same arguments previously made with reference to Hogan *et al.*.

Moreover, Hogan *et al.* transfers IL-12 through a vaccinia virus vector, unlike Li *et al.*, which uses liposomal mediated plasmid transfer of interferon-gamma. Hogan *et al.* and Li *et al.* employ very different methods of gene transfer.

Furthermore, Dow *et al.* teaches away from the composition of the claims, as currently amended. Dow *et al.* states that "traditional naked DNA delivery, which has been touted as having an adjuvant effect, is far less effective than the present compositions at stimulating a non-antigen specific immune response (see column 12, lines 13-17). By contrast, the background of the application notes that the "direct effects of these cytokine plasmids as genetic adjuvants in the allergen vaccines used for AIT have not been addressed." (page 2, lines 12-13). Furthermore, like the Li *et al.* reference, which uses a liposome in one example, Dow *et al.* employs the same, and teaches away from using viral vectors used by Hogan *et al.* by noting that "unlike many protocols for administration of viral vector-based genetic vaccines, the present method can be used to repeatedly deliver the therapeutic composition described herein without consequences associated with some non-specific arms of the immune response, such as the complement cascade (column 12, lines 18-22).

In addition, the O'Donnell *et al.* publication merely observes that intravesical co-administration of BCG plus rIL-12 augments urinary IFN-γ production more strongly than either single agent alone, providing an immunological basis for using exogenous IL-12 in conjunction with BCG for bladder cancer immunotherapy. This observed increase in endogenous IFN-γ production upon co-administration of IL-12 and antigen does not provide a reasonable expectation of success in increasing Th1-type cytokine production and decreasing Th2-type cytokine production by administering a plasmid encoding nucleic acid sequences encoding human IL-12 and human IFN-γ.

With regard to claim 68, the combination of references would not necessarily produce the same predictable results. For example, the Hogan *et al.* reference (see page 415, last paragraph, to page 416, first line) notes that treated mice had IgG2a antibody levels that were similar to those found in controls. This is different from the composition claimed in claim 68, wherein one of the effects is to increase IgG2a. Thus, claims 54-58, and 68 are non-obvious over the cited references. Accordingly, reconsideration and withdrawal of the rejection under 35 U.S.C. §103(a) is respectfully requested.

Claims 2-4, 6-8, 45-48, and 50 have been rejected under 35 U.S.C. §112, first paragraph, as claiming new matter. Claims 2 and 4 have been cancelled. Claims 3, 6, 7, 8, 45-48, and 50 have been amended. Accordingly, this rejection under 35 U.S.C. §112, first paragraph, is rendered moot.

Claims 1-4, 6-9, 11, 12, 14, 15, 18-21, 23-31, 43-50, and 52-68 have been rejected under 35 U.S.C. §112, first paragraph, as non-enabled by the subject specification. The applicants respectfully submit that the claimed invention, as currently amended, is fully enabled by the specification.

Claims 1 and 43 have been amended to recite "....nucleic acid sequence encoding p35 and p40 subunits of human IL-12.. ." Thus, the claims as amended encompass an expression vector that is operable and encodes the p35 and p40 subunits of human IL-12. In addition, the claims as amended encompass a promoter that is operably linked to the gene of interest. Moreover, the claims as amended encompass encoding amino acid sequences that have the biological activity of IL-12 and IFN-γ and thus, results in the Ig and cytokine expression profile claimed.

As the court in *Liebel* recently stated, a "specification need not necessarily describe how to make and use every embodiment of the invention, because the artisan's knowledge of the prior art and routine experimentation can often fill in gaps." *Liebel-Flarsheim Co. v. Medrad*, WL 851205 at *8 (Fed. Cir. 2007), citing *AK Steel Corp. v. Sollac & Ugine,* 344 F.3d 1234, 1244 (Fed. Cir. 2003). Nevertheless, numerous preferred examples of administration are taught in the specification and administration is not necessarily unpredictable.

In addition to intramuscular administration and subcutaneous injection, the specification teaches other forms of administration. For example, page 12, third paragraph, of the specification incorporates U.S. Patent No. 6,489,306 by reference, which describes an example of intranasal administration that may be utilized to administer the nucleic acid sequences claimed in this application. Thus, the applicants need not describe every administration route.

Nevertheless, many references describe successful administration routes in immunology. Roy, in U.S. Patent No. 6,475,995, on column 2, lines 46-49, has noted that *successful immunization* has been demonstrated with administration of plasmid DNA by *intramuscular, intradermal, intravenous and subcutaneous* routes. Wahren and Lu, in their review article, *DNA Vaccines: An Overview*, page 4, last paragraph, have noted that DNA vaccines have been delivered by a variety of routes. Felgner, in U.S. Patent No. 6,710,035, describes many routes of administering plasmids encoding immunogenic peptides which include *intramuscular, intravenous, intranasal, subcutaneous, and intradermal* routes (column 23, line 60 to column 24, lines 1-4; Examples 15-18;

and claims 12-17, for example). Thus, a person of ordinary skill in the art would be able to use well-known successfully used gene delivery routes for immunotherapy.

With regard to the cited references, Van Drunen Littel van den Hurk *et al.* notes that cytokine co-administration might enhance the efficacy of DNA vaccines (see page 119, second paragraph). Scheerlinck also notes that it is clear that cytokines... can be used to modulate DNA vaccines (see page 2653, concluding remarks, first three lines). Although the Examiner cites Gautam *et al.* for the proposition that various barriers to delivery exist, nothing in the Gautam *et al.* reference mentions the use of cytokines for promoting enhanced delivery, where the naked DNA itself includes cytokine nucleotide sequences.

Yang is focused on gene therapy in the cardiovascular system, and has limited relevance to the claimed invention. For example, the cited discussion, regarding problems associated with passive diffusion catheters and poor control of delivery to cells of targeted vessels does not apply. Moreover, Yang notes that most investigations about the imaging of gene therapy involve non-cardiovascular systems, which include the subject matter of the current application (see page 36, fifth paragraph).

With regard to the method of delivery, claims 1 and 43 have been amended to recite the term "co-administering." Support for this amendment can be found at page 5, lines 17-25, and page 8, lines 5-8, of the specification. The method of delivering both cytokines is described and enabled by the specification.

The expression profile of the cytokines in the specification represents the full breadth of Th1 type cytokine production and Th2 type cytokine production. For example, at page 31, lines 5-8, of the specification indicates that IL-12 is the primary determinant of Th1 differentiation, and that endogenously synthesized IFN-γ both accelerates and enhances the Th1 differentiating effects of IL-12. Copies of the Wenner *et al.* (*J Immunol*, 1996, 156:1442-1447) and Bradley *et al.* (*J Immunol*, 1996, 157:1350-1358) publications, which are cited at page 31 of the subject specification for this premise, are submitted herewith.

Glimcher *et al.*, in U.S. Patent No. 6,399,322, in column 2, lines 13-16, notes that IL-4 promotes the differentiation of Th2 cells while IL-12 and interferon-gamma have the opposite effect. For example, administration of recombinant IL-4 or antibodies to IL-12 ameliorate EAE, a Th1-

driven autoimmune disease, while anti-IL-4 antibodies cure a Th2-mediated parasitic disease, *Leishmania major* (column 2, lines 21-27). However, like the applicants (specification, page 2, lines 5-7), Glimcher *et al.* (column 2, lines 28-32) notes that systemic administration of cytokines or antibodies may have unwanted side effects but concludes that alternative approaches to manipulating Th1/Th2 subsets are still needed, which the subject invention, like the Glimcher *et al.* patent, addresses. Accordingly, the expression profiles of the representative Th1 and Th2 cytokines are predictive of the class of the Th1 and Th2 cytokines, as shown by the examples provided. Accordingly, reconsideration and withdrawal of the rejection under 35 U.S.C. §112, first paragraph, is respectfully requested.

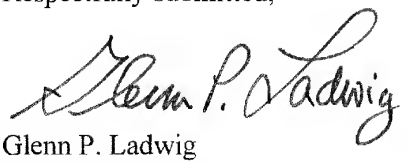
Claims 1-4, 6-9, 11, 12, 14, 15, 18-21, 23-31, 43-50, and 52-68 have been rejected under 35 U.S.C. §112, second paragraph, as indefinite. By this amendment, independent claims 1, 43, and 54 have been amended to make clear that the promoter sequences are operably linked to the nucleic acid sequences encoding the p35 and p40 subunits of human IL-12 and human IFN-γ. Accordingly, reconsideration and withdrawal of the rejection under 35 U.S.C. §112, second paragraph, is respectfully requested.

In view of the foregoing remarks and amendments to the claims, the applicants believe that the currently pending claims are in condition for allowance, and such action is respectfully requested.

The Commissioner is hereby authorized to charge any fees under 37 C.F.R. §§1.16 or 1.17 as required by this paper to Deposit Account 19-0065.

The applicants invite the Examiner to call the undersigned if clarification is needed on any of this response, or if the Examiner believes a telephonic interview would expedite the prosecution of the subject application to completion.

Respectfully submitted,



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Bradley *et al.* (*J Immunol*, 1996, 157:1350-1358)
Supplemental Information Disclosure Statement; Form PTO/SB/08; references

Roles of IFN-γ and IFN-α in IL-12-Induced T Helper Cell-1 Development[1]

Cynthia A. Wenner,* Mehmet L. Güler,* Steven E. Macatonia,† Anne O'Garra,† and Kenneth M. Murphy[2]*

IL-12 and IL-4 direct T cell development toward Th1 and Th2 phenotypes, respectively. While IFN-γ and IFN-α have been reported to regulate Th1 development as well, the mechanism and cellular locus of their effects are unclear. In this study, we use a TCR-transgenic system to examine the actions of these cytokines on $CD4^+$ T cell phenotype development. We find that neither IFN-γ nor IFN-α can induce Th1 development alone. However, IFN-γ can significantly augment IL-12 priming for subsequent IFN-γ production by T cells. Interestingly, lymphocyte endothelial cell adhesion molecule-1^{bright} (naive) T cells require IFN-γ during primary activation for maximal IL-12-induced Th1 development, whereas lymphocyte endothelial cell adhesion molecule-1^{dull} (memory) T cells do not. IFN-α only partially substitutes for IFN-γ in promoting IL-12-induced Th1 development. When the endogenous IFN-γ present in primary T cell cultures is neutralized, IFN-α treatment augments IL-12-induced effects on inhibition of subsequent IL-4 production, but fails to significantly enhance IL-12 priming for subsequent IFN-γ production. Thus, our data suggest that IFN-γ provides a direct costimulatory signal to T cells to up-regulate IL-12-induced Th1 development and may operate by inducing IL-12 responsiveness in naive T cells. *The Journal of Immunology,* **1996, 156: 1442–1447.**

T helper phenotype development can be critical in specific pathogen resistance (1, 2). In murine experimental leishmaniasis, the development of Th1 responses confers resistance to pathogens, whereas the development of Th2 responses permits lethal pathogen dissemination (3–6). IL-12 and IL-4 critically influence development of naive T cells toward Th1 and Th2 subsets, respectively (7–15). Despite the importance of IL-12 and IL-4, other cytokines also participate in controlling Th development of naive T cells. IL-10 and IFN-γ can contribute to T cell development through inhibition or activation of APCs (16–18). While IL-10 acts primarily to inhibit APC expression of costimulators and cytokines (9, 16, 19, 20), the locus of action of IFN-γ may include the T cell as well as the APC (12, 21).

IFN-γ may influence Th1 development by both indirect and direct pathways. IFN-γ enhances macrophage production of IL-12 in response to pathogens (22, 23), indirectly promoting Th1 development. While not sufficient for directing Th1 development alone, IFN-γ may also act directly on naive T cells to allow IL-12-induced Th1 responses. First, in an APC-dependent system of T cell development, neutralization of IFN-γ blocked IL-12-mediated Th1 induction (24). Second, neutralization of IFN-γ in APC-independent systems blocked IL-12-induced Th1 development (12, 21, 25, 26). However, using TCR-transgenic T cells sorted for CD4 and Vα11, but not sorted for lymphocyte endothelial cell adhesion molecule (LECAM)-1[3] expression, neutralization of IFN-γ was found not to block Th1 development in an accessory cell-dependent system (12). In addition, IFN-γ-deficient mice produce effective anti-viral cytolytic immune responses (27), and IFN-γR-deficient mice produce Th1 type cells despite an increased susceptibility to *Leishmania major* (28). Thus, the precise contribution of IFN-γ in Th1 development requires clarification.

In addition, IFN-α has been reported to influence Th1 development. Addition of IFN-α increased the frequency of human cytolytic $CD4^+$ T cells (29, 30) and suppressed T cell help for B cell Ab production (31, 32). However, these studies did not directly address the mode of action or cellular locus of IFN-α in Th phenotype development. Therefore, the potential role of IFN-α in regulating Th1 development requires further analysis.

In the present study, we examined the interactions of IFN-α and IFN-γ with IL-12 for promoting Th1 development. Our results show that IFN-γ can act directly on naive T cells to promote IL-12 responsiveness for Th1 development, perhaps by induction of the IL-12R components. Furthermore, we show that IFN-α cannot, as previous studies suggested, directly induce Th1 development, but may indirectly promote Th1 development through augmenting subsequent inhibition of IL-4 production by IL-12. These results clarify previous contradictory observations regarding the separate roles of these cytokines in Th1 development.

Materials and Methods

Animals

Five- to ten-week-old female BALB/c mice were purchased from Harlan Sprague-Dawley (Indianapolis, IN) or from Simonsen Labs (Gilroy, CA). DO11.10 TCR-α/β-transgenic mice in the BALB/c genetic background have been described (33). DO11.10 TCR-transgenic mice were backcrossed onto the B10.D2 background for three generations for analysis shown in Figure 1.

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[2] Address correspondence and reprint requests to Dr. Kenneth M. Murphy, Department of Pathology, Washington University School of Medicine, St. Louis, MO 63110.

[3] Abbreviations used in this paper: LECAM, lymphocyte endothelial cell adhesion molecule; SAPE, streptavidin-phycoerythrin.

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Table I. *IL-12-induced Th1 development is inhibited by neutralization of IFN-γ*[a]

Primary Stimulation Condition		Secondary IFN-γ Production[b]	
IL-12	Anti-IFN-γ	24 h	48 h
0	0	<4	<4
5	0	876	2127
5	10	378 ± 19	651 ± 174
5	50	314 ± 4	498 ± 51
60	0	995	2335
60	10	459 ± 32	771 ± 53
60	50	288 ± 2	416 ± 36

[a] DO11.10 transgenic T cells were purified by cell sorting to greater than 98% CD4$^+$ LECAM-1$^+$ expression and stimulated at 2.5 × 10^5/well with OVA peptide (0.3 μM) and irradiated TA3 cells (5 × 10^5/well) in the absence or presence of IL-12 (5 or 60 U/ml) and anti-IFN-γ mAb H22 (0, 10, or 50 μg/ml). T cells were harvested on day 7, washed twice and 2.5 × 10^5 T cells were restimulated. Supernatants were harvested at 48 h and assayed by ELISA for IFN-γ as described in the *Materials and Methods*.

[b] Results are given as the concentration or mean concentration (U/ml) ± SD of duplicate secondary stimulation determinations.

Tissue culture media

Iscove's modified Dulbecco's Eagle medium or RPMI were supplemented as described (24, 34). Dendritic cells were isolated in complete RPMI 1640 Dutch modification medium (Life Technologics, Inc., Grand Island, NY) (24).

Cytokines, mAbs, and flow cytometry reagents

Reagents used included recombinant murine IL-12 (Hoffmann-La Roche, Nutley, NJ, or as described in Ref. 24 for Fig. 2), human IFN-$\alpha_{A/D}$ (Hoffmann-La Roche, Geneva, Switzerland), recombinant murine IFN-γ (Genentech, San Francisco, CA), anti-IL-4 mAb 11B11 as described (35), hamster anti-mouse IFN-γ mAb H22 and sheep anti-mouse IFN-αβ Ig Ab 1-9F (provided by Dr. Ion Gresser, Laboratory of Viral Oncology, CNRS, Villejuif Cedex, France), neutralizing IFN-γ mAb XMG1.2 (described previously in Ref. 36), and anti-IL-12 mAb TOSH (provided by Dr. C. S. Tripp and Dr. E. R. Unanue, Washington University School of Medicine, St. Louis, MO). Murine-specific reagents for flow cytometry included anti-CD4-FITC, anti-LECAM-1-PE, anti-CD3-FITC, anti-CD4-Tricolor, streptavidin-phycoerythrin (SAPE), and avidin Texas Red (PharMingen, San Diego, CA).

Cell purification

Enriched dendritic cells from normal BALB/c splenocytes were purified by flow cytometry as N418^{high} Mac-1^{low} (37). T cells were isolated by density gradient (Histopaque-1119; Sigma Chemical Co., St. Louis, MO), depleted of MHC class II$^+$ and CD8$^+$ cells with anti-class II mAb CA4 (a gift from Dr. A. L. Glasebrook, Eli Lily, Indianapolis, IN), anti-CD8 mAb 3.155 (38), and rabbit and guinea pig complement (Life Technologies, Inc.), depleted of sIg$^+$ cells by sheep anti-mouse IgG magnetic beads (Dynal, Lake Success, NY), and depleted of adherent cells by incubation at 37°C for 2 h on plastic. These enriched T cells were at least 95% CD4$^+$ and 80% KJ1-26$^+$ (Fig. 3) (39). For more highly purified preparations (Figs. 1, 2, 4, 5, and Table I), DO11.10$^+$ T cells were first enriched by density gradient separation and complement lysis of MHC class II$^+$ and CD8$^+$ cells, stained with anti-CD4-FITC and anti-LECAM-1-PE, and purified by cell sorting (FACS Vantage; Becton Dickinson, San Jose, CA) to >98% LECAM-1^{bright} CD4$^+$ T cells. For use in experiments with dendritic cells (Fig. 2), T cells were stained with CD4-Tricolor and LECAM-1-PE mAbs and purified into CD4$^+$, LECAM-1^{bright} CD4$^+$, and LECAM-1^{dull} CD4$^+$ populations on a FACStar^{Plus} (Becton Dickinson).

T cell cultures

Purified T cells (2.5 × 10^5/well) were stimulated in 2-ml cultures in 24-well plates with 0.3 μM OVA and irradiated APCs: BALB/c splenocytes (2,000 rad, 5 × 10^6/well); H-2$^{d/k}$ B cell-B lymphoma hybridoma TA3 (10,000 rad, 5 × 10^5/well) (40); or BALB/c dendritic cells (1,500 rad, 1 × 10^4/well). Supernatants were collected at 48 h post-primary stimulation, and cells expanded threefold into fresh medium at 72 h. T cells were harvested on day 7, washed twice, and restimulated at 2.5 × 10^5/well and 5 × 10^6 BALB/c or 5 × 10^5 TA3 APC/well with 0.3 μM OVA. Th phenotypes were determined by cytokine determinations from 24- and 48-h supernatants (9). Primary activation included the following cytokines: IL-12 (5 or 60 U/ml), IL-4 (100 U/ml), IFN-γ (50 to 300 U/ml), IFN-α (3 to 300 U/ml), anti-IL-12 mAb (TOSH, 3 μg/ml), anti-IL-4 11B11 mAb (10 μg/ml), anti-IFN-α/β polyclonal Ab 1-9F (1:4000), and anti-IFN-γ mAb H22 (10 or 50 μg/ml) or XMG1.2 (10 μg/ml).

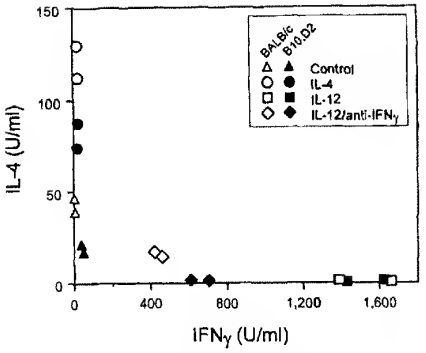

FIGURE 1. IFN-γ augments IL-12-induced Th1 development in both BAL8/c and B10.D2-derived T cells. DO11.10-transgenic splenic T cells from BALB/c and B10.D2 background mice were purified by cell sorting to greater than 98% CD4$^+$ LECAM-1$^+$ expression. A total of 2.5 × 10^5 T cells/well were stimulated with 0.3 μM OVA peptide and 5 × 10^6 irradiated BALB/c splenocytes (2000 rad) in the presence of the indicated experimental conditions: control (no additions); IL-12 (5 U/ml); IL-4 (100 U/ml); or IL-12 (5 U/ml) + anti-IFN-γ (H22, 30 μg/ml). T cells were harvested on day 7, washed twice, and 2.5 × 10^5 T cells were restimulated without other additions. Supernatants were harvested at 48 h and assayed by ELISA for IL-4 and IFN-γ to assess the effects of the indicated primary conditions on Th phenotype development. Duplicate data points represent two independent primary cultures assayed under identical conditions in the same experiment. Data are representative of three separate experiments.

Cytokine assays

IL-2, IL-4, and IFN-γ were quantitated by ELISA as described (9, 41). IL-2 and IL-4 standards were calibrated to cytokines purchased from Genzyme (Cambridge, MA). IFN-γ standards were provided by Dr. R. D. Schreiber (Washington University School of Medicine, St. Louis, MO). The 0.1 ng/ml IFN-γ standard is equivalent to 1 U/ml (41).

Results

IFN-γ augments IL-12-induced Th1 development of naive T cells in vitro

To evaluate the distinct roles for various cytokines in promoting Th1 and Th2 development, we used DO11.10 TCR-transgenic mice as a source of Ag-specific T cells for studies in vitro. To examine the effects of IFN-γ on Th1 development in an APC-dependent system, we used irradiated, TA3 B cell hybridoma cells (40) for analysis of T cell development in vitro (11). We chose TA3 cells, in addition to splenocytes and dendritic cells, as APCs since we have demonstrated that these cells provide costimulation for T cell growth but do not mediate heat-killed *Listeria monocytogenes* (HKLM)-induced Th1 development (11). Addition of IL-12 to naive T cells isolated from BALB/c mice induced strong Th1 development when added to primary cultures at either 5 or 60 U/ml (Table I). The presence of IFN-γ during primary stimulation significantly augmented IL-12-induced Th1 development, since neutralizing IFN-γ with H22 Ab substantially diminished IL-12-induced IFN-γ production in a dose-dependent manner (Table I).

Because the genetic background of T cells (BALB/c vs B10.D2) can significantly influence the default pathway of T cell development (39), we investigated the possibility that IFN-γ affects Th1

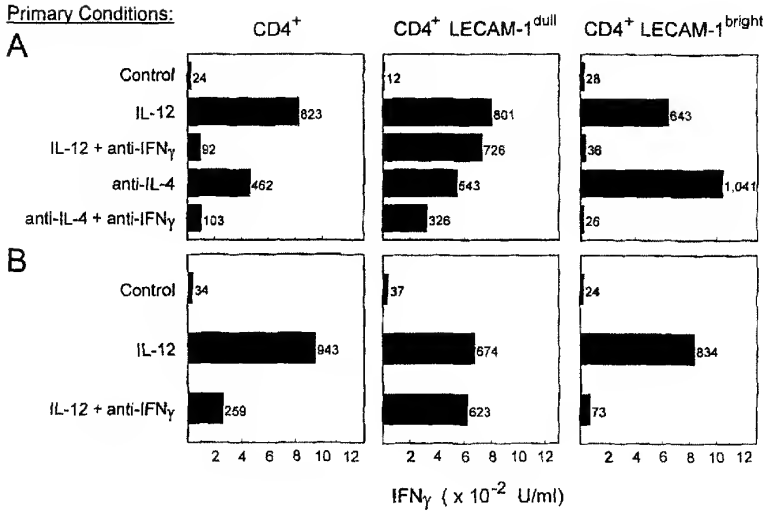

FIGURE 2. IFN-γ augments IL-12-induced Th1 induction in naive T cells, but not LECAM-1^{dull} T cells. T cells were purified as described in Figure 1 to obtain CD4$^+$, CD4$^+$ LECAM-1^{dull}, and CD4$^+$ LECAM-1^{bright} populations. A total of 2.5×10^5 cells/well were activated with OVA with either no additions (control), or with the addition of IL-12 (60 U/ml), anti-IL-4 (10 μg/ml), or anti-IFN-γ (XMG1.2, 10 μg/ml) as indicated in the figure, using either (*A*) irradiated dendritic cells (1500 rad) or (*B*) irradiated BALB/c splenocytes (2000 rad) as APCs. Cells were restimulated on day 7, and 48-h supernatants were analyzed for cytokines by ELISA. Results presented are representative of data obtained in four separate experiments.

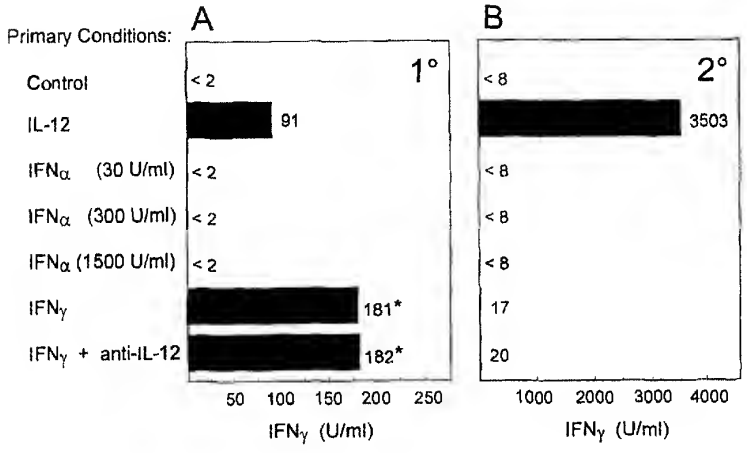

FIGURE 3. IFN-α is insufficient for induction of Th1 development. Enriched DO11.10 TCR-transgenic CD4$^+$ T cells (2.5×10^5/well) were activated with OVA and irradiated BALB/c splenocytes in the presence of medium alone (control) or with the addition of IL-12 (5 U/ml), IFN-α at the concentration indicated in the figure, or IFN-γ (300 U/ml) in the presence or absence of a neutralizing IL-12 mAb (TOSH, 3 μg/ml). *A*, Cytokine production in supernatants harvested 48 h after primary activation was measured by ELISA. IFN-γ production in IFN-γ-treated conditions (*) reflects exogenously added cytokine. *B*, T cells were restimulated on day 7 following primary activation without additions and cytokines in 48-h supernatants measured by ELISA.

development differentially in T cells derived from distinct genetic backgrounds (Fig. 1). Addition of IL-12 to naive T cells from either BALB/c or MHC haplotype-matched B10.D2 mice induced strong Th1 development (Fig. 1). IFN-γ neutralization significantly inhibited IL-12-induced priming for subsequent IFN-γ production in both BALB/c and B10.D2-derived T cells (Fig. 1). However, IL-12-induced Th1 development was not absolutely dependent on IFN-γ, since IFN-γ neutralization in the presence of IL-12 induced a more Th1-like response in comparison with Th0 responses developing with no exogenous cytokine additions (Fig. 1). Thus, IFN-γ can augment IL-12-induced Th1 development in naive T cells from both strains, implying a general mechanism present in distinct genetic backgrounds. This result extends our earlier report that IL-12-induced Th1 development was dependent on IFN-γ when using dendritic cells as APCs (24).

Since other earlier studies suggested no effect of IFN-γ on APC-dependent Th1 development (12, 42, 43), we addressed a potential source of experimental differences between these studies. We suspected that contamination of primary T cells by memory Th1 cells may contribute to IFN-γ-independent IL-12-induced Th1 cytokine production. The LECAM-1^{dull} surface phenotype identifies memory T cells that have undergone previous activation (44). Thus, we purified naive and memory populations of CD4$^+$ T cells from unimmunized DO11.10-transgenic mice by cell sorting and determined the requirements for Th1 development of the LECAM-1^{bright} and LECAM-1^{dull} T cell populations using dendritic cells (Fig. 2*A*) or splenocytes (Fig. 2*B*) as APCs. Maximal development of IFN-γ-producing Th1 cells from LECAM-1^{bright} T cells required both IFN-γ and IL-12 during the primary culture, since neutralization of IFN-γ substantially diminished development of IFN-γ-producing T cells. In contrast, LECAM-1^{dull} T cells did not show this effect, and developed into a Th1 population producing similar levels of IFN-γ either with or without IFN-γ during in vitro activation (Fig. 2). This difference between naive and memory T cells in the augmentation of Th1 development by IFN-γ was further illustrated by inducing Th1 development using the anti-IL-4 Ab in the presence and absence of IFN-γ. LECAM-1^{bright} T cells developed a Th1 phenotype when IL-4 was neutralized in the primary activation only when endogenous IFN-γ was present (Fig. 2*A*). In contrast, LECAM-1^{dull} T cells developed into a Th1 phenotype, regardless of whether IFN-γ was neutralized during in vitro activation. Therefore, unsorted populations of T cells used in some previous studies (12, 42, 43) may contain memory T cells and consequently would show less dependence on IFN-γ addition in primary cultures for optimal IL-12-induced Th1 development.

Differential effects of IFN-α and IFN-γ on promoting T cell responses to IL-12

We previously have shown that IFN-γ alone does not induce Th1 development (11). Because of the reported effects of IFN-α on Th1/Th2 development (29–32), we tested whether IFN-α alone was sufficient for Th1 induction. We activated TCR-transgenic T cells in primary cultures in the presence of either IL-12, IFN-α, or IFN-γ, and examined immediate IFN-γ production and effects on Th1 development (Fig. 3). IFN-α was unable to enhance IFN-γ production by T cells either in primary cultures throughout a range of concentrations (Fig. 3*A*), or to direct the development of IFN-γ-producing Th1 cells (Fig. 3*B*). IFN-γ added in the primary stimulation (Fig. 3*A*) either with or without IL-12-neutralizing mAb (Fig. 3*A*) also failed to induce Th1 development (Fig. 3*B*). In contrast, the positive control IL-12 addition induced IFN-γ production during both primary and secondary activation. Thus, IFN-α and IFN-γ are both individually unable to induce development of Th1 cells.

As a positive control for these effects, we measured MHC class I expression in unactivated $CD4^+$ T cells left untreated or treated with IFN-α for 3 days (Fig. 4). Less than 10% of untreated T cells expressed high levels of surface MHC (Fig. 4*A*). In contrast, both IFN-γ (Fig. 4*B*) and IFN-α (Fig. 4, *C* and *D*) induced approximately 70% MHC class I^{high}-expressing T cells, confirming IFN-α activity in these cells. Thus, the inability of IFN-α to induce Th1 development was not due to an inability of these cells to recognize and signal in response to IFN-α.

IFN-α and IFN-γ both activate Stat1, and thus partially overlap in their signaling pathway (45). Therefore, we asked whether IFN-α might provide a similar role as IFN-γ in the induction of Th1 development by IL-12. Neutralization of endogenous IFN-γ significantly diminished IL-12-induced IFN-γ production to a level fivefold lower than that observed upon addition of IFN-γ and IL-12 to give a maximal Th1 response (Fig. 5*A*). In contrast, IFN-α did not mimic IFN-γ in promoting Th1 development, since addition of increasing doses of IFN-α in the absence of endogenous IFN-γ did not restore IL-12-induced IFN-γ production (Fig. 5*A*).

We also examined developmental effects of IFN-γ and IFN-α on IL-4 production. IL-12 treatment in the presence of neutralizing Abs to both IFN-γ and IFN-αβ during primary activation decreased IL-4 production in comparison with development under the control condition (no exogenous cytokine addition, Fig. 5*B*). Thus, priming of naive T cells in the presence of IL-12 leads to decreased IL-4 production independently of IFN-γ or IFN-α. However, IFN-α, like IFN-γ, augmented inhibition of IL-4 production, since addition of IFN-α to primary cultures in the presence of IL-12 and anti-IFN-γ mAb enhanced IL-12-induced IL-4 inhibition in a dose-dependent manner (Fig. 5*B*). Therefore, IFN-α partially mimics the effect of IFN-γ on Th1 development by promoting subsequent inhibition of IL-4 production in response to IL-12. However, IFN-α does not substitute for IFN-γ in significantly augmenting IL-12-induced priming for IFN-γ production.

Discussion

The aim of this study was to evaluate the interaction between IFN-α, IFN-γ, and IL-12 for effects on T helper development.

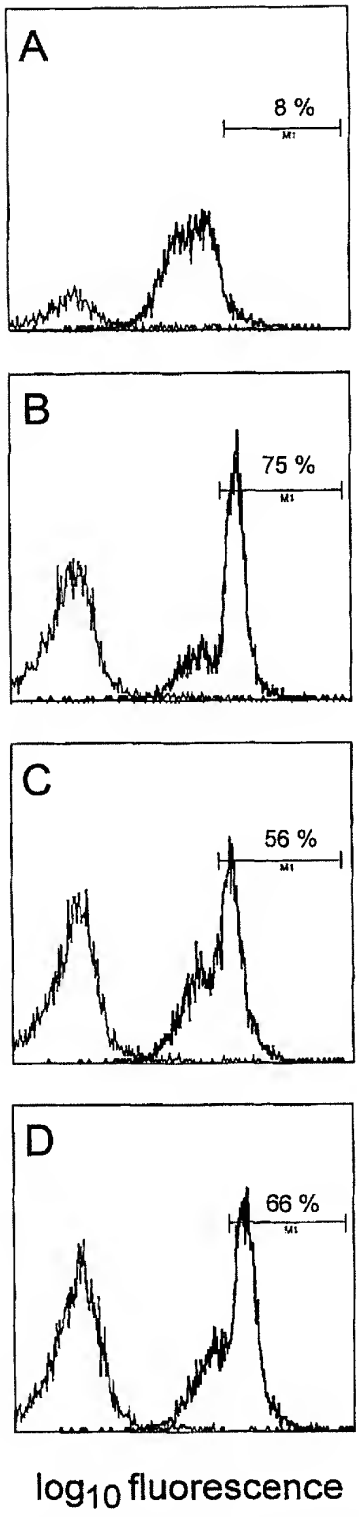

FIGURE 4. IFN-α induces class I MHC expression in TCR-αβ-transgenic $CD4^+$ T cells. Enriched DO11.10 TCR-transgenic $CD4^+$ T cells (1×10^6/well) were treated for 3 days in the presence of (*A*) medium alone, (*B*) IFN-γ (300 U/ml), (*C*) IFN-α (30 U/ml), or (*D*) IFN-α (300 U/ml), and analyzed by flow cytometry following staining with anti-CD4-FITC and anti-H-$2K^d$-Biotin/SAPE (bold) or anti-CD4-FITC and SAPE alone (normal) as a negative control. Histograms are gated for $CD4^+$ T cells and show the level of expression of class I MHC (H-$2K^d$) for each condition. Numbers above the marker are the percentage of cells within the indicated gate and represent high class I MHC expression.

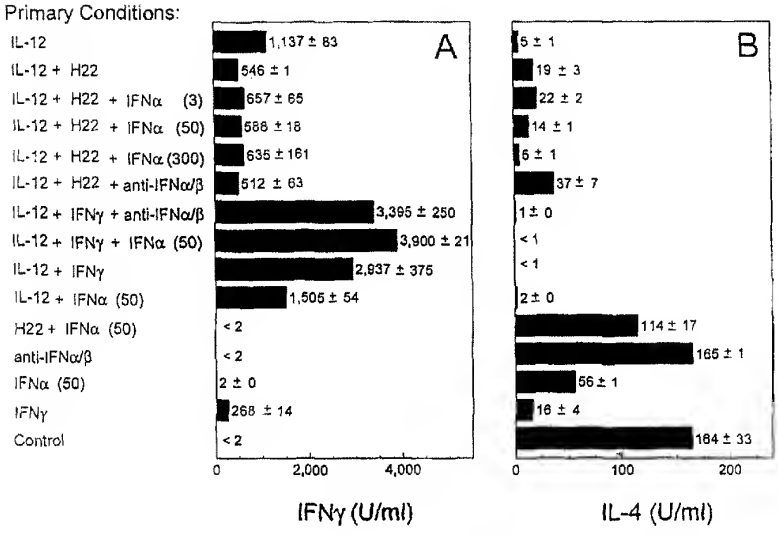

FIGURE 5. IFN-α and IFN-γ are not interchangeable in augmentation of IL-12-induced Th1 development. LECAM-1^{bright} CD4$^+$ T cells (2.5×10^5/well) were stimulated with OVA and irradiated TA3 B cell hybridoma cells (10,000 rad; 5×10^5/well) in the presence of the indicated primary conditions: 5 U/ml IL-12; 50 μg/ml anti-IFN-γ mAb H22; 50 U/ml IFN-γ; 3, 50, or 300 U/ml IFN-α as indicated; and 1:4000 anti-IFN-α/β. T cells were restimulated on day 7 in the absence of added conditions, and 48-h supernatants were analyzed for (*A*) IFN-γ or (*B*) IL-4 levels by ELISA. Data are presented as the average concentration ± SD of duplicates. Data are representative of five experiments analyzing development of sorted LECAM-1^{bright} T cells.

Hsieh et al. (9–11), Macatonia et al. (24), and others (21, 25, 46) have suggested that IL-12 induction of Th1 development may be influenced by costimulation with IFN-γ, whereas other studies have not observed this IFN-γ effect (12, 42, 43). In this report, we clarify this difference by showing that the influence of IFN-γ on IL-12 responsiveness is evident in naive T cells, but not in memory T cells. Thus, we show that neutralization of IFN-γ during in vitro stimulation significantly inhibits IL-12 priming for IFN-γ production by LECAM-1^{bright} CD4$^+$ T cells, but not by LECAM-1^{dull} CD4$^+$ T cells. We have recently shown that the IL-12 signaling pathway is developmentally regulated during Th differentiation (47). Specifically, we found that induction of Th2 development accompanied a rapid loss in IL-12 signaling capacity, allowing early Th2 cells to resist phenotype reversal by IL-12 (47). Naive T cells lack IL-12R function, but activated, presumably memory, T cells express functional IL-12Rs (48, 49). Those results, taken together with the data presented here, suggest that IFN-γ may enhance the expression of functional IL-12Rs by naive T cells.

Both IFN-γ and IFN-α increase the frequency of cytolytic Th0 and Th1 human T cells (29, 30). However, other studies reported that IFN-γ alone is not sufficient to direct Th1 development (11, 21, 24, 25). We show that IFN-α alone also fails to induce the Th1 phenotype. Since some earlier studies preceded the identification of IL-12's role in Th1 development (29, 32, 50), potential interactions between IFN-α, IFN-γ, and IL-12 may have been overlooked. This is the first report to address the roles of IFN-α and IFN-γ while considering interactions with IL-12.

IFN-γ and IFN-α partially overlap in their effects on gene induction. For example, either IFN-γ or IFN-α can provide the IFN requirement for LPS-induced nitric oxide synthase expression in macrophages (51, 52). Both IFN-γ and IFN-α induce the transcription factor IRF-1, which is required for activation of the inducible nitric oxide synthase gene (53). Another common effect of IFN-γ and IFN-α signaling is up-regulation of MHC class I surface expression (54). In contrast, IFN-γ, but not IFN-α, can induce MHC class II expression in macrophages (54). Here, we show that IFN-γ can substantially strengthen IL-12-induced Th1 development, both enhancing IL-12 priming for IFN-γ production and inhibiting subsequent IL-4 production, while IFN-α synergizes with IL-12 only for the subsequent inhibition of IL-4 (Fig. 5). Thus, because the signaling pathways activated by IFN-α and IFN-γ are not identical, it is conceivable that some of their effects will overlap only partially. For example, while IFN-γ and IFN-α could each act to inhibit IL-4 production, only IFN-γ appears to significantly prime for subsequent IFN-γ production.

We show that naive T cells can develop a Th1 phenotype in response to IL-12 and IFN-α in the absence of IFN-γ, although the level of IFN-γ is sub-optimal (Fig. 5). This finding is consistent with published reports that Th1 cells can develop in mice in which the IFN-γ gene (27) or the IFN-γR gene (28) has been disrupted. Here, we show that the actions of IFN-α are likely to involve regulation of IL-4 levels rather than directly controlling IL-12 responsiveness and IFN-γ production. Since IL-12 has been shown here to act independently of IFN-α and IFN-γ in memory T cells (Fig. 2), it will be important to determine whether the expression of functional IL-12Rs is controlled by IFN-γ and is different between naive and memory T cells and to identify the mechanism of IL-12 responsiveness in the complete absence of IFN-γ. Interestingly, since memory T cells can show similar modes of regulation by cytokines as naive T cells in vivo (as shown recently by Bradley, Yoshimoto, and Swain (55)), it will be important to pinpoint the mode of action of IFN-γ for its apparent actions on naive, but not memory, T cells.

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A Direct Role for IFN-γ in Regulation of Th1 Cell Development[1]

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IL-12 has been identified as a major cytokine influencing the differentiation of CD4 cells to a Th1 phenotype, whereas a role for IFN-γ is controversial. We investigated the interrelationship between IL-12 and IFN-γ in promoting Th1 responses using naive CD4 cells reactive with pigeon cytochrome *c* from TCR transgenics and memory CD4 cells derived by in vivo priming with KLH. Without exogenous rIL-12 or rIFN-γ, primary and memory effectors induced by Ag or anti-CD3 and anti-CD28 secreted variable levels of IL-2 and IFN-γ. The level of IFN-γ secreted by effectors correlated with endogenous IFN-γ produced in primary cultures, and anti-IFN-γ largely inhibited the development of effectors producing IFN-γ. With optimal TCR stimulation and costimulation, endogenous IFN-γ, without IL-12, was sufficient to elicit Th1 cells via an autocrine mechanism, whereas with suboptimal stimulation, exogenous rIFN-γ or rIL-12 was required for Th1 development. However, rIL-12 was more effective than rIFN-γ, partially because rIL-12 greatly enhanced autocrine production of IFN-γ, and optimal development of the Th1 phenotype was mediated by the synergistic actions of both cytokines. Thus, both IFN-γ and IL-12 can independently regulate Th1 development, but because of IFN-γ-mediated feedback, their relative contributions are determined by the conditions of T cell stimulation. The extent of differentiation to a Th1 phenotype may, therefore, depend on the availability of both APC-derived IL-12 and autocrine IFN-γ consequent to the overall strength of T cell stimulation. *The Journal of Immunology,* **1996, 157: 1350–1358.**

It is now well recognized that induction of cell-mediated vs humoral immune responses largely correlates with development of Th1 and Th2 helper T cell subsets, respectively (1–3), which can determine resistance vs susceptibility to disease. Th1 cells produce IL-2, IFN-γ, TNF-α, and TNF-β, whereas Th2 cells secrete IL-4, IL-5, IL-6, IL-10, and IL-13. In general, cytokines present during the initiation of a response can direct the development of CD4 subsets, and repeated exposure to Ag together with particular arrays of cytokines influences the degree of polarization that ensues (4, 5). IL-12 and IL-4 have been identified as key cytokines that direct preferential development of Th1 or Th2 responses, respectively. IL-12 produced by macrophages (6, 7) and other phagocytic cells (8, 9) as well as by dendritic cells (10) early in an inflammatory response to infection induces differentiation of naive CD4 cells to a Th1 phenotype. While potential sources of IL-4 for differentiation of Th2 cells include basophils, mast cells, and NK1.1$^+$ T cells (11, 12), the production of IL-4 by naive CD4 cells shortly after activation can also regulate Th2 development (13, 14), suggesting that the early cytokine response of CD4 cells themselves may play a crucial role in determining the polarization of developing effectors.

Although IL-12 is the major cytokine that induces the development of Th1 cells, many of the effects associated with IL-12 may be attributable to the induction of IFN-γ (reviewed in Ref. 15). Thus, it is unclear to what extent IFN-γ produced by CD4 cells regulates the development of Th1 cells or if it is simply an effector molecule produced from an array of cells that include CD4, CD8, and NK cells in response to IL-12 (16–19). Some studies suggest that IFN-γ has no direct role in CD4 differentiation, with the absence of IL-4 during effector development apparently being sufficient to promote Th1 cell development and addition of rIFN-γ being without effect (20–22). However, others studies show that rIFN-γ substantially enhances the development of Th1 effectors, even when endogenous IL-4 is neutralized (23–26). Under certain conditions, induction of IFN-γ secretion by CD4 cells in response to IL-12 does appear to be IFN-γ dependent (17, 22, 26). These observations suggest that both cytokines may be required for optimal Th1 induction, but their respective roles in the regulation of Th1 development are unclear.

In this study, we critically evaluated the contributions of IFN-γ and IL-12 to the development of Th1 cells. We compared the effects of these cytokines on the induction of Th1 cells from resting naive and memory CD4 cells. Memory cells differ from naive cells by their capacity to secrete cytokines other than IL-2 almost immediately after activation, but both populations develop into similar effector cells under the influence of cytokines (11, 14, 24, 27–29). We used both Ag-specific (APC-dependent) and polyclonal (APC-independent) models that we have extensively characterized (24, 30–32) to evaluate the respective roles of IFN-γ and IL-12 independently of contributions of cytokines from activated T cells or from APC in various experimental designs. Our results conclusively demonstrate that IFN-γ alone can direct differentiation of Th1 cells from either naive or memory CD4 cells from mice of the C57BL/6 and B10.BR backgrounds. Moreover, endogenous IFN-γ secretion in response to stimulation appears to contribute to the development of a Th1 phenotype in these strains. Our results indicate that in the absence of APC-derived IL-12, IFN-γ produced by CD4 cells may be critical for the initial polarization to a Th1

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phenotype, playing a role analogous to that of autocrine IL-4 in the induction of Th2 cells.

Materials and Methods

Mice

C57BL/6 mice were purchased from The Jackson Laboratory (Bar Harbor, ME). (B10.BR × C57BL/6)F1 or B10.BR, Vβ3/Vα11 TCR transgenic mice (33) and H-2^b IFN-γ-KO mice that were backcrossed onto the C57BL/6 background (34) were bred in our facility at University of California-San Diego. Adult mice of the same age and sex were used in individual experiments.

Recombinant cytokines and anti-cytokine Abs

Murine rIL-2 was obtained from the culture supernatants of X63.Ag8–653 cells transfected with murine cDNA for the respective cytokines (35). Murine rIFN-γ was purchased from Life Technologies (Grand Island, NY). rIL-12 (sp. act., 5.6×10^6 U/mg) and polyclonal sheep anti-mouse IL-12 were generously provided by Dr. Stanley Wolf (Genetics Institute, Cambridge, MA). The following anti-cytokine Abs were purified from ascites: 7D4 and PC.61 (anti-IL-2R), 11B11 (anti-IL-4), XMG1.2 and R46A2 (anti-IFN-γ), and TRFK4 and TRFK5 (anti-IL-5).

CD4 cell preparation

For experiments involving Ag-specific responses, naive CD4 cells were isolated from the spleens from 2- to 4-mo-old TCR transgenic mice. Memory CD4 cells were obtained from C57BL/6 mice immunized at 6 to 8 wk of age by i.p. injection of 100 μg of KLH[4] (Calbiochem, La Jolla, CA), precipitated with 1.8 mg of alum, and injection of 10^9 *B. pertussis* vaccine organisms and subsequently thymectomized 1 mo later. Mice were used >25 wk after thymectomy when only cells of memory phenotype remain, with <5% contamination by cells of naive phenotype as judged by the lack of surface L-selectin (36). CD4 cells were enriched as previously described (14, 24). Briefly, spleen cells were passaged over nylon wool columns to deplete B cells, macrophages, dendritic cells, and activated T cells. CD8 cells and any residual B cells were then depleted using a mixture of monoclonal anti-CD8 (HO 2.2 and 3.155), anti-class II (D3.137, CA4, and M5114), and anti-HSA (J11D), followed by treatment with a mouse anti-rat κ Ab (MAR 18.5) and by a mixture of rabbit and guinea pig complement (Life Technologies). Resting, high density CD4 cells were isolated by discontinuous Percoll gradient centrifugation (four layers: 45%, 53%, 62%, and 80%) at the interface of the 80 and 62% layers. This procedure removes any residual low density/activated cells including effector CD4 cells that also exhibit surface marker expression characteristic of memory cells. Finally, any remaining macrophages or dendritic cells were removed by adherence. The resulting populations were 95 to 98% CD4 cells that were comprised exclusively of resting cells, as indicated by forward scatter and lack of expression of IL-2R (7D4) (14, 24).

In some experiments, indicated in the text, naive and memory CD4 cells were isolated from normal mice by magnetic separation (magnetic activated cell sorting) on the basis of differential expression of L-selectin as previously described (28, 31). Briefly, following depletion of B cells and CD8 cells, enriched CD4 cells were sequentially stained with biotinylated anti-L-selectin (MEL-14), FITC-streptavidin (Zymed, San Francisco, CA), and biotinylated magnetic beads (Miltenyi Biotech, Sunnyvale, CA). Positively staining cells were retained by the columns in the presence of a magnetic field, whereas the negative population was eluted. Populations were checked for purity by flow cytometry and reincubated on the column as necessary to achieve >95% separation of positive and negative subsets.

APC

For experiments with naive TCR transgenic CD4 cells, a fibroblast line transfected with I-E^k and ICAM-1 was used as APC (DCEK-ICAM) (14, 32) to present peptide 88–104 of pigeon cytochrome *c* (PCCF). For responses of memory CD4 cells, APC were spleen cells from unimmunized mice depleted of T cells using a mixture of anti-Thy.1.2 (F7D5 and HO.13.14), anti-CD8 (HO.2.2), and anti-CD4 (RL.172.4) and complement. APC were pulsed with 100 μg/ml KLH (Calbiochem, La Jolla, CA) for 18 h in 100-mm petri dishes (Fisher Scientific, Pittsburgh, PA) at 5×10^6/ml. The medium was RPMI 1640 (Irvine Scientific, Santa Ana, CA) supplemented with 7 to 10% FCS (HyClone Laboratories, Logan, UT), 200 μg/ml penicillin, 200 U/ml streptomycin, 4 mM L-glutamine, 10 mM HEPES, and 5×10^{-5} M 2-ME. All APC were treated for 30 min at 37°C with 50 μg/ml mitomycin C (Sigma Chemical Co., St. Louis, MO) before use.

Generation of effector Th

Effectors were generated by either Ag-specific or polyclonal stimulation of CD4 cells for 4 days in 1.0- or 2.0-ml volumes (in 48- or 24-well plates, respectively; Costar, Cambridge, MA). Cultures were supplemented with recombinant cytokines and anti-cytokine Abs as indicated in the text for individual experiments. The optimal doses of cytokines were 20 U/ml rIL-2, 1000 to 2000 U/ml rIFN-γ, and 2 to 4 ng/ml rIL-12. To block endogenous cytokine secretion, 10 μg/ml of anti-IFN-γ (XMG1.2), anti-IL-12, or anti-IL-4 (11B11) were used. For Ag-specific responses, naive cells were cultured at 2 to 3×10^5/ml with 5 μM PCCF, purified by HPLC from whole pigeon cytochrome *c* (Sigma Chemical Co.), and as APC, an equal number of T-depleted spleen cells or 5×10^4/ml DCEK-ICAM cells. Memory cells were cultured at 3 to 5×10^5/ml with 5×10^5/ml KLH-pulsed T-depleted spleen cells. After 4 days, the cells were harvested, washed, and restimulated in duplicate or triplicate cultures (depending on cell recovery) at the concentrations indicated in the text. For restimulation, naive cells were cultured with 1 to 2×10^5/ml DCEK-ICAM and 5 μM PCCF in 0.5 ml; memory cells were cultured with 1×10^6/ml KLH-pulsed APC in 0.25 to 0.5 ml. For polyclonal responses, effector cells were generated by culturing CD4 cells at 2 to 5×10^5/ml in 0.5- to 1.0-ml volumes in 48-well plates that were coated with protein G-purified anti-CD3 (2C11) in 0.3 ml PBS for 2 h at 37°C and then washed. Doses of 0.2 to 20 μg/ml anti-CD3 were used, as indicated in the text for individual experiments. To provide costimulation, an ascites preparation of anti-CD28 Ab, 37.51 (a generous gift from Dr. J. Allison, University of California-Berkeley) was used at dilutions ranging from 1/400 to 1/2000, as described in the text. After 4 days, CD4 cells were restimulated at 1 to 5×10^5/ml in anti-CD3-coated plates in medium containing anti-CD28. For all experiments, supernatants were harvested from replicate cultures 18 h after restimulation of effectors, pooled, and stored at −20°C. The proliferation of effectors upon restimulation was measured by incorporation of [^{3}H]thymidine for 20 to 26 h as previously described (14, 32).

Measurement of cytokine production by CD4 cells

IL-2 and IL-4 were detected by bioassay as previously described (28, 36) by measuring proliferation of the NK cell line (37), which responds to both cytokines. The assay is rendered specific for IL-2 when IL-4 activity is blocked by the presence of 11B11 Ab, and specific for IL-4 when IL-2 activity is blocked by the addition of anti-IL-2R Abs (7D4 and PC.61). The data are expressed as half-maximal units, and serial dilutions of test supernatants, assayed in duplicate or triplicate, are referenced to recombinant cytokines where 1 U of IL-2 equals 14 pg of protein, and 1 U of IL-4 equals 0.7 pg of protein. Variations in replicate cultures were <10% of the mean counts per minute. IFN-γ and IL-5 were detected by ELISA using R46A2 or TRFK5, respectively, as coating Abs, and biotinylated-XMG1.2 or -TRFK4, respectively, as second step reagents. IFN-γ or IL-5 was quantitated by comparison of serial dilutions of test supernatants in duplicate wells to standard curves using recombinant cytokines and are expressed as units per milliliter, where 1 U of IFN-γ equals 0.1 ng of protein and 1 U of IL-5 equals 3.1 ng of protein.

Analysis of cytokine mRNA by reverse transcription-PCR

In brief, enriched splenic CD4 cells were separated into L-selectin-positive and -negative subsets and then cultured at 5×10^5/ml, as described above, for 4 days with rIL-2 and with 10 μg/ml plate-bound anti-CD3 and soluble anti-CD28 (1/500). Total RNA was obtained by guanidine isothiocyanate, phenol-chloroform extraction, and ethanol precipitation. cDNA was prepared, as described previously (14, 38), from 5×10^5 cells of each subset before culture (0 h) and at 12, 24, 36, and 48 h. Aliquots of cDNA corresponding to 5×10^4 cells were amplified for 30 cycles using the following conditions for IFN-γ: 94°C for 2 min, 55°C for 2 min, and 72°C for 1 min. The sequences of the oligonucleotides used for PCR were: IFN-γ sense primer, 5′-TTTGAAGTCTTGAAAGACAATCAG-3′; and IFN-γ antisense primer, 5′-GCAGCGACTCCTTTTCCGCTTCCT-3′. PCR products were separated by electrophoresis on 2% agarose gels and transferred to nylon membranes (CUNO Laboratory Products, Meridan, CT). The Southern blots were labeled with a ^{32}P-labeled IFN-γ-specific probe (644 bp *Pst* fragment from clone pmc14), and IFN-γ was visualized by autoradiography after 24 h.

[4] Abbreviations used in this paper: KLH, keyhole limpet hemocyanin; ICAM-1, intracellular adhesion molecule-1; PCCF, pigeon cytochrome *c*.

Results

Effects of rIFN-γ and rIL-12 on development of CD4 effectors that secrete IFN-γ and IL-2

To evaluate the roles of IL-12 and IFN-γ in regulating the development of Th1 effector populations, we compared their abilities to induce IFN-γ-secreting effectors from resting naive and memory CD4 cells stimulated either by Ag and APC or by anti-CD3 and anti-CD28 in the absence of APC. Highly purified CD4 cells were isolated by passage over nylon wool columns followed by complement-dependent cytotoxicity, Percoll density gradient centrifugation, and adherence as described in *Materials and Methods*. Naive CD4 cells were obtained from mice transgenic for a TCR (Vα11 and Vβ3) specific for a peptide fragment of pigeon cytochrome *c* (PCCF 88–104) (33). The majority (95%) of the CD4 cells from these mice express the transgene and bear surface markers characteristic of naive cells (L-selectin^{high}, CD45RB^{high}, and CD44^{low}) (30, 31). Memory CD4 cells were isolated from mice that were primed with KLH, thymectomized, and left for >6 mo. At this time, T cells of naive phenotype and function are depleted (39), and memory T cells are correspondingly enriched (36, 40).

To induce Ag-specific responses, naive CD4 cells were cultured with PCCF and fibroblast APC-expressing B7–1 and ICAM-1 along with I-E^k (DCEK-ICAM). We have previously shown that these cells are highly effective APC (14, 32), inducing responses from PCCF-specific CD4 cells equivalent to those obtained with dendritic cells as APC (41). In corresponding cultures, memory CD4 cells were cultured with T cell-depleted spleen cells that were pulsed with KLH as APC. To elicit polyclonal responses, naive or memory CD4 cells were stimulated with 10 to 20 μg/ml of immobilized anti-CD3. Since we and others have previously shown that naive CD4 cells have an absolute requirement for costimulation (14, 32, 42), and memory CD4 cells respond optimally when costimulation is provided (30), soluble anti-CD28 at a 1/1000 dilution was added to all cultures. To generate effectors, cultures were supplemented with optimal doses of murine rIL-2 (20 U/ml) and either rIFN-γ (1000–2000 U/ml) or rIL-12 (2–4 ng/ml). In most of the experiments shown, endogenous IL-4 (if any) was neutralized by the addition of 10 μg/ml anti-IL-4 Ab, although the effects shown did not depend on blocking IL-4 since similar experiments in the absence of anti-IL-4 produced identical results. After 4 days, the cells were harvested and recultured in the absence of exogenous cytokines, but with the same stimuli as those used in the initial phase of incubation. IFN-γ secretion into culture supernatants was measured 18 h later.

As shown in Figure 1, effectors generated from either naive (*A*) or memory (*B*) CD4 cells by either Ag-specific or polyclonal stimulation when only exogenous rIL-2 was added produced moderate levels of IFN-γ, demonstrating the predisposition toward a Th1 phenotype of CD4 cells from mice on the C57BL/6 and B10.BR backgrounds (43). Inclusion of rIFN-γ during the development of effector populations resulted in modest but consistent increases (1.5- to 2-fold) in IFN-γ secretion by effectors following restimulation compared with levels produced by effectors induced in the presence of IL-2 only. Similar results were consistently obtained in six additional experiments.

In contrast to the modest effects of rIFN-γ on the development of Th1 effectors, rIL-12 consistently induced dramatic increases in the capacity of both primary (Fig. 1*A*) and memory (Fig. 1*B*) CD4 effector populations to produce IFN-γ in response to restimulation. This enhancement ranged from 4- to >20-fold in five experiments with naive cells and seven experiments with memory cells. The results in the APC-independent model indicate that IFN-γ and IL-12 act directly on CD4 cells to promote IFN-γ secretion. In addition, the effects of both IFN-γ and IL-12 on the development of effector populations from resting naive and memory cells were remarkably similar.

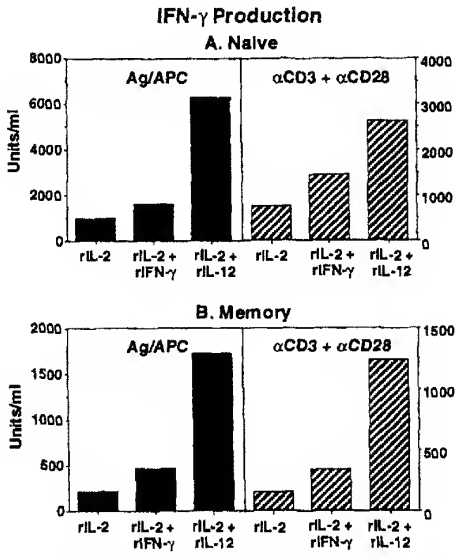

FIGURE 1. Effects of exogenous IFN-γ and IL-12 on the development of naive and memory CD4 effectors with the capacity to secrete IFN-γ. CD4 cells were purified as indicated in *Materials and Methods* from the spleens of PCCF-specific TCR (C57BL/6 × B10.BR)F_1 or B10.BR transgenic mice (naive; *A*) or from KLH-primed, adult thymectomized C57BL/6 mice (memory; *B*). For Ag stimulation, naive CD4 cells were cultured at 2 × 10^5/ml with 5 μM PCCF and 0.5 × 10^5/ml DCEK-ICAM cells as APC. Memory CD4 cells were cultured at 5 × 10^5/ml with 10 × 10^5/ml KLH-pulsed T cell-depleted spleen cells as APC. For polyclonal stimulation, resting CD4 cells were isolated from the spleens of normal C57BL/6 or B10.BR mice, separated into L-selectin$^+$ (naive) and L-selectin$^-$ (memory) subsets by magnetic sorting, and cultured at 1 × 10^5/ml with 1/1000 anti-CD28 in plates coated with 10 μg/ml anti-CD3. To all cultures were added 20 U/ml murine rIL-2, 10 μg/ml anti-IL-4, and either 1000 to 2000 U/ml murine rIFN-γ or 2 to 4 ng/ml murine rIL-12. After 4 days, the cells were restimulated at 2 × 10^5/ml in the absence of rIL-2, but with the same stimuli as those used in the initial incubation. IFN-γ secretion was measured 18 h later. Similar results were obtained in six additional experiments regardless of whether endogenous IL-4 production was blocked.

Since the secretion of IL-2 in addition to IFN-γ is usually associated with Th1 cells, we also evaluated the effects of exogenous rIFN-γ and rIL-12 on the capacity of primary and memory CD4 effectors to secrete IL-2 (Fig. 2). Much lower levels of IL-2 were consistently detected in supernatants from memory effector cells stimulated with Ag/APC compared with those stimulated with anti-CD3 and anti-CD28 (Fig. 2*B*), but otherwise effectors generated from naive and memory CD4 cells showed similar patterns of response to rIFN-γ and rIL-12. In contrast to the enhancing effects of exogenous rIFN-γ and rIL-12 on the capacity of effectors to secrete IFN-γ, inhibition of IL-2 secretion by restimulated effectors was observed. For naive CD4 cells, a reduction of IL-2 secretion from effectors that developed in the presence of rIFN-γ was consistently observed in four experiments (20 to 80%), regardless of whether CD4 cells were stimulated with Ag or, in the absence of APC, with anti-CD3 and anti-CD28 (Fig. 2*A*). Primary effectors generated in the presence of rIL-12 exhibited an even greater reduction in the capacity to produce IL-2, with 60 to 90% lower

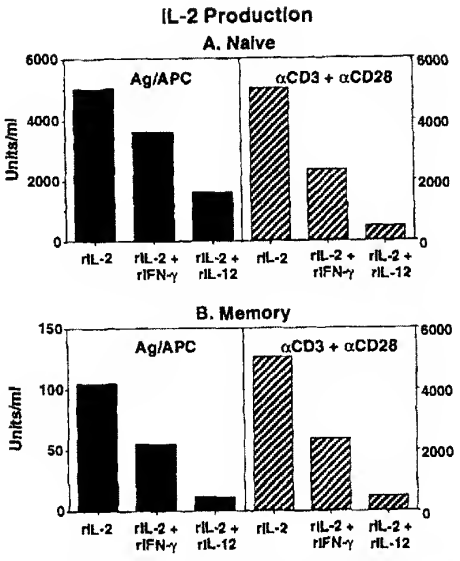

FIGURE 2. Effect of exogenous IFN-γ and IL-12 on the development of naive and memory CD4 effectors with the capacity to secrete IL-2. Naive cells (*A*) and memory cells (*B*) obtained from TCR transgenic mice or adult thymectomized normal mice were stimulated as described in Figure 1. For Ag-specific responses, naive and memory cells were cultured with PCCF and DCEK-ICAM or with KLH-pulsed T-depleted spleen cells, respectively. For polyclonal stimulation, immobilized anti-CD3 and soluble anti-CD28 were used. Cultures were supplemented with 20 U/ml rIL-2, 1000 to 2000 U/ml rIFN-γ, or 4 ng/ml rIL-12. After 4 days, the cells were restimulated at 2×10^5/ml in the absence of rIL-2, but with the same stimuli as those used in the initial incubation. IL-2 secretion was measured 18 h later. Comparable results were obtained with both naive and memory cells in four additional experiments with each population. The proliferation of T cells in these cultures did not vary significantly (e.g., for naive CD4 cells stimulated with PCCF and DCEK-ICAM, the proliferation of effectors upon restimulation, as measured by incorporation of [^{3}H]thymidine for 20 to 26 h, was 17,129 ± 355 cpm for cells generated with rIL-2, 13,551 ± 232 cpm for those with rIFN-γ, and 25,835 ± 971 cpm for those with rIL-12.

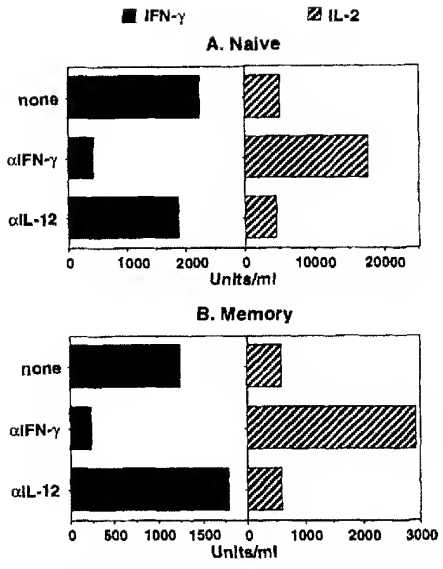

FIGURE 3. Effect of endogenous IFN-γ on the development of effectors from naive and memory CD4 cells. Resting CD4 cells from (C57BL/6 × B10.BR)F_1 or B10.BR TCR transgenic mice were separated into naive and memory cells on the basis of L-selectin expression and stimulated at 2×10^5/ml with 10 μg/ml plate-bound anti-CD3 and 1/2000 anti-CD28. To these cultures were added 20 U/ml murine rIL-2 (none) or IL-2 with either anti-IFN-γ (10 μg/ml) or anti-IL-12 (10 μg/ml). After 4 days, the cells were restimulated at 2×10^5/ml with the same concentrations of initial stimuli in the absence of cytokines or anti-cytokine Abs. IFN-γ in culture supernatants was measured after 18 h. Comparable results were obtained in five experiments.

levels than controls. Comparable results were obtained in four experiments with memory CD4 cells, exemplified by representative data depicted in Figure 2*B*. Because supernatants were tested by 18 h after restimulation, and no differences in proliferation between naive CD4 cells treated with rIFN-γ or rIL-12 and controls were observed (see Fig. 2), it is unlikely that variations in the consumption of IL-2 accounts for the observed reduction in the amounts of IL-2 in supernatants. Thus, an increased capacity of effectors to secrete IFN-γ correlated with a decreased capacity to produce IL-2. The data indicate that both IFN-γ and IL-12 directly regulate the patterns of cytokines that are produced by CD4 effectors generated from naive or memory cells.

Effect of endogenous IFN-γ on development of effectors from naive and memory CD4 cells

To evaluate whether endogenous IFN-γ secretion contributes to Th1 polarization of CD4 effector populations derived from mice on the C57BL/6 or B10.BR backgrounds, anti-IFN-γ-blocking Abs or, as a control, anti-IL-12-blocking Abs were included in primary cultures of resting naive or memory CD4 cells that were stimulated with immobilized anti-CD3 and anti-CD28 in the presence of rIL-2, as described above. The secretion of IFN-γ and IL-2 was assessed 18 h after restimulation (in the absence of rIL-2 or blocking Abs). Primary and memory effectors that developed in the absence of anti-cytokine Abs secreted both IL-2 and IFN-γ (Fig. 3, *A* and *B*, respectively). Primary effectors produced very low or undetectable IL-4 and IL-5, while memory effectors secreted low to moderate levels of these cytokines unless anti-IL-4 was present during effector generation (not shown). Inclusion of anti-IFN-γ Ab during effector generation, in a dose capable of neutralizing 5000 U/ml of IFN-γ, markedly diminished their capacity to secrete IFN-γ (fivefold) and correspondingly increased their capacity to secrete IL-2 (three- to fivefold).

As expected in the absence of APC, addition of anti-IL-12 to primary cultures, in a dose capable of completely neutralizing the effects of >30 ng/ml rIL-12, had no significant effect on the capacity of effectors to secrete either IFN-γ or IL-2. Neither anti-IFN-γ nor anti-IL-12 had any effect on IL-4 or IL-5 secretion by effectors (not shown). Similar results were obtained in two additional experiments, in which blocking of endogenous IFN-γ in primary cultures caused decreases in IFN-γ secretion ranging from three- to eightfold and corresponding increases in IL-2. Comparable results were also obtained in three experiments in which naive and memory effectors were induced by Ag and APC (not shown), indicating that production of IL-12, if any, by the APC populations used to elicit effectors did not contribute to their development. These data demonstrate that for CD4 cells from mice from C57BL/6 and B10.BR backgrounds, IFN-γ, in the absence of IL-12, can account for polarization of CD4 effectors to a Th1 cytokine pattern via an autocrine mechanism.

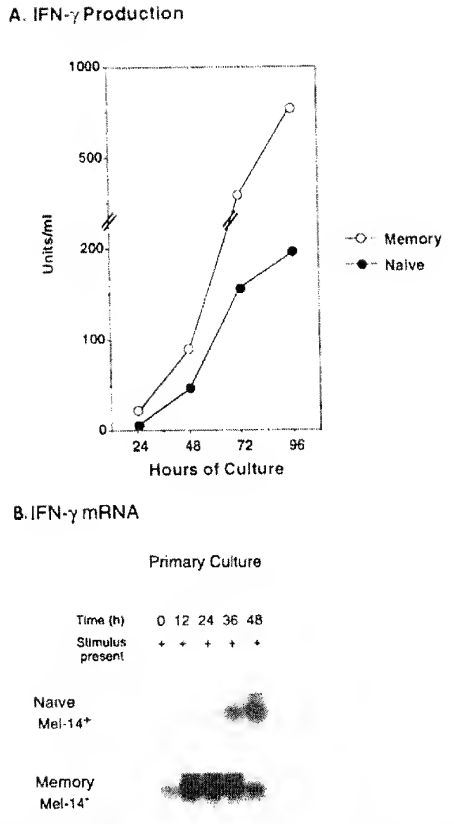

FIGURE 4. Autocrine production of IFN-γ from naive and memory CD4 cells. Resting CD4 cells from normal (C57BL/6 × B10.BR)F1 mice were separated into naive and memory cells on the basis of L-selectin expression and stimulated at 1×10^6/ml with 10 μg/ml plate-bound anti-CD3 and a 1/1000 dilution of anti-CD28. *A*, IFN-γ in supernatants was measured at 24-h intervals throughout the 4 days of primary culture to generate effectors. *B*, Total RNA was extracted from 5×10^5 CD4 cells before culture and from replicate cultures 12, 24, 36, and 48 h after stimulation. cDNA was prepared, and aliquots corresponding to 5×10^4 cells were amplified by PCR using primers for IFN-γ. PCR products were separated by electrophoresis and visualized by autoradiography of Southern blots that were labeled with a ^{32}P-labeled IFN-γ-specific probe. Synthesis of IFN-γ was not apparent in cultures unless a source of stimulation was provided.

Autocrine production of IFN-γ from naive and memory CD4 cells

Although resting memory CD4 cells have the capacity to secrete low levels of several cytokines shortly after activation (14, 28, 29), naive CD4 cells produce exclusively IL-2 and must further differentiate to acquire the capacity to secrete additional cytokines (14, 29). Since endogenous IFN-γ appeared to play a role in the development of Th1 effector cells from both naive and memory CD4 cells (Fig. 4), we measured IFN-γ secretion and mRNA synthesis by CD4 cells in response to initial stimulation in primary cultures. Purified CD4 cells from normal (C57BL/6 × B10.BR)F1 mice were separated into naive and memory cells on the basis of L-selectin expression (14) and stimulated with optimal concentrations of plate-bound anti-CD3 (10 μg/ml) and anti-CD28 (1/1000). In Figure 4*A*, IFN-γ in supernatants was measured at 24-h intervals throughout the 4 days of primary culture to generate effectors. In Figure 4*B*, total RNA was extracted from CD4 cells before culture and from replicate cultures 12, 24, 36, and 48 h after stimulation. The appearance of IFN-γ message was evaluated by reverse transcription PCR and Southern blots.

The results shown in Figure 4*A* demonstrate that secretion was detectable in primary cultures of both naive (closed symbols) and memory (open symbols) CD4 cells. However, IFN-γ production by memory cells was substantially greater and measurable 24 h earlier than that by naive cells. As shown in Figure 4*B*, IFN-γ mRNA was only detectable 24 h after stimulation of the naive population, whereas IFN-γ message was strongly induced within 12 h in memory cells. No IFN-γ mRNA was detected by culture in the absence of stimulation (not shown). The results demonstrate that although naive CD4 cells lag behind memory CD4 cells, within 24 h of stimulation they acquire the capacity to produce IFN-γ independently of contributions from APC. These results suggest that both subsets have the potential to produce sufficient IFN-γ in response to activation to influence effector development toward a Th1 phenotype.

Effect of strength of stimulus on IFN-γ-induced development of Th1 effectors

The finding that endogenous secretion of IFN-γ by naive and memory CD4 cells can polarize developing effectors to a Th1 phenotype (Fig. 3) suggests that low levels of IFN-γ may be sufficient to achieve this effect. Thus, the often limited ability of exogenous rIFN-γ to enhance the development of Th1 effectors in this (Fig. 1) and previous studies (17, 22, 26) may reflect variations in endogenous IFN-γ secretion by CD4 cells during effector generation under different conditions. To investigate whether altering conditions of CD4 cell activation would influence the magnitude of the exogenous rIFN-γ effect on induction of Th1 effectors, naive or memory CD4 cells were stimulated in the presence or the absence of rIFN-γ, using either suboptimal (0.2 μg/ml anti-CD3 and 1/5000 anti-CD28) or optimal (10 μg/ml anti-CD3 and 1/400 anti-CD28) levels of stimulation, as determined previously (30, 32). As shown in Figure 5*A*, when weaker stimulation was used, the addition of exogenous rIFN-γ increased the capacity of both primary and memory effectors to secrete IFN-γ by 5- to 10-fold. In contrast, when strong stimulation was used to induce effectors, the addition of rIFN-γ had only a limited effect on their ability to secrete IFN-γ (Fig. 5*B*), which was even less pronounced than that shown in Figure 1, where 2.5-fold less anti-CD28 was used. Similar results were observed in two additional experiments. These data suggest that the potency of stimulation influences the magnitude of autocrine IFN-γ secretion by CD4 cells, and that sufficient levels are attained under conditions of strong stimulation to drive the development of Th1 cells in the complete absence of IL-12.

At 24-h intervals, we also measured IFN-γ recovered in culture supernatants of naive and memory CD4 cells induced with optimal vs suboptimal stimulation. As shown in Figure 5*B*, the levels of IFN-γ recovered from supernatants of both naive and memory cells were somewhat greater with optimal stimulation (open triangles) than with suboptimal stimulation (open circles), although by 4 days, the effect of stimulation was greater with memory (5-fold) than naive cells (1.4-fold). Previous studies have demonstrated that rIL-12 induces T cells to secrete IFN-γ (16, 17, 19, 23), but whether this effect is immediate, and whether both naive and memory CD4 cells respond similarly are unknown. Therefore, we compared the effects of rIL-12 on IFN-γ secretion by CD4 effector cells induced with strong vs weak stimulation.

The data presented in Figure 5*B* demonstrate that the presence of an optimal dose of rIL-12 for effector generation (4 ng/ml) dramatically increased the levels of IFN-γ present in culture supernatants by 3 days (6- to 16-fold; closed symbols), particularly

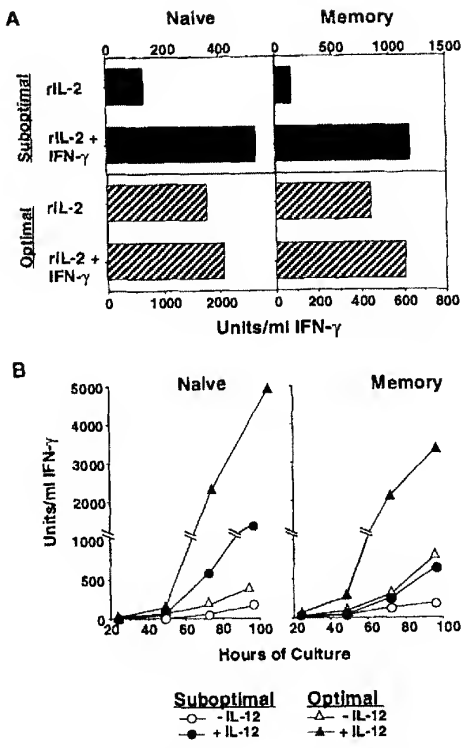

FIGURE 5. Effect of strength of stimulus on IFN-γ-induced development of Th1 effectors. Resting CD4 cells from normal (C57BL/6 × B10.BR)F1 mice were separated into naive and memory cells on the basis of L-selectin expression. *A*, Cells were stimulated at 2 × 10^5/ml with either 0.2 μg/ml immobilized anti-CD3 and a 1/5000 dilution of anti-CD28 (suboptimal) or 10 μg/ml immobilized anti-CD3 and a 1/400 dilution of anti-CD28 (optimal). Cultures were supplemented with 20 U/ml rIL-2 or with rIL-2 and 1000 U/ml rIFN-γ. After 4 days, cells were washed and restimulated at 2 × 10^5/ml with 10 μg/ml plate-bound anti-CD3 and a 1/1000 dilution of anti-CD28. Supernatants were tested for IFN-γ after 18 h. *B*, Naive (L-selectin$^+$) and memory (L-selectin$^-$) cells were cultured with either suboptimal (0.2 μg anti-CD3 and 1/5000 dilution of anti-CD28) or optimal (10 μg/ml anti-CD3 and 1/400 dilution of anti-CD28) concentrations of stimuli at 1 × 10^5/ml with either rIL-2 alone or with rIL-2 and 4 ng/ml rIL-12. IFN-γ in supernatants was measured at 24-h intervals throughout the 4 days of primary culture. Similar results were obtained in two additional experiments.

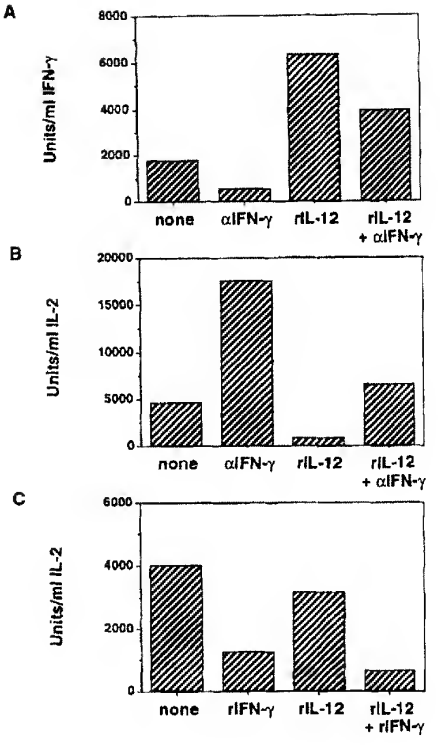

FIGURE 6. IL-12 effects are partially mediated by IFN-γ. In *a* and *b*, Th1 effectors were induced from resting naive CD4 cells from PCCF-specific TCR transgenic mice that were stimulated for 4 days at 2 × 10^5/ml with immobilized anti-CD3 (10 μg/ml), anti-CD3 and soluble anti-CD28 (1/1000) in the presence of 20 U/ml rIL-2, 10 μg/ml anti-IL-4, and either 10 μg/ml anti-IFN-γ, 4 ng/ml rIL-12, or both. The cells were restimulated at 2 × 10^5/ml in the absence of cytokines or anti-cytokine Abs, and IFN-γ (*A*) or IL-2 (*B*) in culture supernatants was measured at 18 h. Comparable results were obtained in three experiments. *C*, Resting CD4 cells isolated from IFN-γ knockout mice were stimulated under the same conditions as those in *a* in the presence of 20 U/ml rIL-2 and either 1000 U/ml rIFN-γ, 4 ng/ml rIL-12, or both. The cells were restimulated at 2 × 10^5/ml in the absence of added cytokines or anti-cytokine Abs. IL-2 in culture supernatants was measured at 18 h. Similar data were obtained in one other experiment.

when strong stimulation was used (closed triangles). With optimal stimulation, even by 24 h, IL-12 induced a 3-fold increase in IFN-γ recovered from memory cell cultures (19 U/ml without rIL-12 compared with 59 U/ml with rIL-12), whereas an increase of similar magnitude was not observed until 48 h with naive cells. Interestingly, higher levels of IFN-γ accumulated in the culture supernatants of naive than in memory CD4 cells, suggesting either greater production of IFN-γ in response to rIL-12 by naive cells or greater utilization of endogenous IFN-γ by memory CD4 cells. Cell recoveries from cultures that received optimal vs suboptimal stimulation were comparable regardless of rIL-12, suggesting that differences in the numbers of cells secreting IFN-γ during culture did not account for the IL-12 effect, but, rather, that IL-12 dramatically augmented IFN-γ production by CD4 cells. Similar results were observed in two additional experiments with polyclonal stimulation as well as in two experiments with memory CD4 cells in response to Ag. The results demonstrate that IL-12 directly affects IFN-γ production by both naive and memory CD4 subsets, and in particular, with strong stimulation, remarkably high levels of IFN-γ are produced.

IL-12 effects are partially mediated by IFN-γ

To investigate whether IFN-γ secreted in response to rIL-12 contributes to the development of effector cells with the capacity to produce IFN-γ, Th1 effectors were induced from naive CD4 cells that were stimulated with optimal levels of immobilized anti-CD3 and soluble anti-CD28 with the addition of anti-IFN-γ, rIL-12, or both. When endogenous IFN-γ secretion was blocked, the development of effectors with the capacity to secrete IFN-γ in response to rIL-12 was diminished by 40% (Fig. 6*A*), and at the same time, the inhibitory effect of IL-12 on IL-2 production was ameliorated, resulting in an eightfold increase in the level of IL-2 (Fig. 6*B*). Comparable results were obtained with memory CD4 cells (not shown) and in two experiments of identical design. Similar data

were also obtained when Ag and APC were used to generate effectors from naive and memory CD4 cells (not shown). Inhibition of IFN-γ secretion by effectors generated in the presence of rIL-12 but in the absence of endogenous IFN-γ ranged from 40 to 70%, while IL-2 responses were augmented by two- to eightfold under these conditions. The results indicate that IFN-γ secreted by CD4 cells in response to rIL-12 is used in an autocrine manner and that the effects of rIL-12 are partially IFN-γ dependent. The data demonstrate that both IL-12 and IFN-γ are needed for optimal development of effector populations that produce high levels of IFN-γ and also suggest that the high levels of IFN-γ induced by IL-12 that accumulate in primary cultures during effector generation down-regulate the capacity of effectors to secrete IL-2.

To delineate the effects of IL-12 and IFN-γ on the development of CD4 effectors independently of endogenous secretion of IFN-γ from CD4 cells, we compared the effects of rIL-12 and rIFN-γ on the development of IL-2-secreting effectors from CD4 cells from IFN-γ knockout mice (34) (Fig. 6*C*) or from wild-type littermates (not shown). The CD4 cells were unable to secrete IFN-γ or synthesize IFN-γ mRNA in response to stimulation (not shown). Effectors were induced from resting, splenic CD4 cells (which contained 20% cells of a memory phenotype) by stimulation with optimal concentrations of anti-CD3 and anti-CD28 in cultures supplemented with rIL-2 and anti-IL-4, and either no further additions (none) or with rIL-12, rIFN-γ, or both cytokines, as indicated in Figure 6*C*. Unlike previous experiments in which the presence of rIFN-γ during effector generation was less effective than IL-12 in reducing the capacity of effectors to produce IL-2 (Fig. 2), when effectors were generated from CD4 cells isolated from IFN-γ knockouts, rIFN-γ, rather than rIL-12, exhibited the more potent down-regulatory effect on IL-2 secretion. However, the most dramatic inhibition was achieved when both cytokines were used in combination during effector development. When endogenous IL-4 was not blocked, effectors generated in the absence of either rIL-12 or rIFN-γ produced moderate levels of IL-4 (267 U/ml) and high levels of IL-5 (1893 U/ml). This suggests that in the IFN-γ KO mice, unlike normal C57BL/6 mice, Th2 effectors develop as a consequence of autocrine IL-4. Under these conditions, neither rIFN-γ nor rIL-12 altered the capacity of effectors to produce Th2 cytokines. Comparable results were obtained in one additional experiment.

Discussion

In this study we critically evaluated the roles of IFN-γ and IL-12 in the development of Th1 effector populations from resting CD4 cells. We compared the effects of these cytokines on rigorously purified naive and memory cells both in Ag-driven models and in responses in the complete absence of APC induced by direct TCR stimulation with immobilized anti-CD3 with costimulation by anti-CD28. Our results conclusively demonstrate that IFN-γ, in the absence of IL-12, directly induced the development of effectors with the capacity to secrete IFN-γ (Fig. 3). IL-12 also induced differentiation of IFN-γ-secreting effector cells independently of IFN-γ by direct effects on CD4 cells (Fig. 6). During effector generation, IL-12 elicited high levels of IFN-γ secretion by CD4 cells shortly after activation in the absence of APC (Fig. 5*B*), and the effects of IL-12 on the development of Th1 cells were in part mediated by endogenous IFN-γ (Fig. 6). These results corroborate previous studies which have indicated that although IL-12 exerts effects that are independent of IFN-γ, induction of Th1 cells is reduced when IFN-γ is blocked (17, 23, 26) or absent (44).

In previous studies of effector development from naive CD4 cells, blocking of endogenous IL-4 was found to be sufficient to promote the differentiation of Th1 cells (6, 20, 21) in at least some circumstances due to the production of low levels of IL-12 by APC that included dendritic cells (10) and macrophages (26). Consistent with reports that the genetic background of murine inbred strains influences the development of Th subsets (43), our results showed the Th1 pattern of cytokines to be the apparent default phenotype from naive CD4 cells from the C57BL/6 and B10.BR strains used in this study, regardless of whether endogenous IL-4 was blocked. Secretion of both IFN-γ and IL-2 was seen upon restimulation (Fig. 3), but little or no IL-4 or IL-5 was found (not shown). As expected from our previous studies (24), memory effectors that were generated without blocking endogenous IL-4 secretion produced low to moderate levels of IL-4 and IL-5 (not shown). Moreover, blocking of IL-4 was sufficient to allow development of Th1 effectors from memory cells.

These patterns of cytokine production by effectors were unaltered by anti-IL-12, suggesting that under the conditions employed, IL-12 secretion did not account for Th1 development in either our Ag-driven or polyclonal models. Instead, the results indicate that IFN-γ secretion by the CD4 population itself in response to activation mediated the development of Th1 effectors (Figs. 3, 4, and 5*B*). In the absence of IL-12, both naive and memory CD4 subsets produced IFN-γ following initial activation (Fig. 4). Although detectable secretion by naive cells lagged behind that of memory cells (Fig. 4*A*; as did synthesis of IFN-γ mRNA, Fig. 4*B*), it is obvious that within a relatively short period of time after stimulation, both naive and memory cells have the capacity to produce IFN-γ in quantities potentially sufficient to drive Th1 development. It is, therefore, possible that in certain infections, endogenous IFN-γ secretion during effector generation would be adequate to promote CD4 development to a Th1 phenotype by autocrine usage without a requirement for IL-12.

Our finding that IFN-γ secreted by CD4 cells can by itself account for induction of Th1 effectors suggests that IFN-γ has a role in Th1 development parallel to that of IL-4 in Th2 development. In response to repetitive stimulation, naive cells can produce enough IL-4 for autocrine regulation of development toward Th2 effectors (14). However, naive cells initially secrete IL-2 following activation (14, 29, 31), and IL-4 synthesis, like that of IFN-γ, only occurs 24 h or more after stimulation (14). While memory cells have the capacity to produce low levels of both IL-4 and IFN-γ almost immediately after activation, they also initially secrete predominantly IL-2 (28–30). The results, therefore, suggest that memory and naive cells are similar in some respects, with both CD4 subsets containing cells that are uncommitted and multipotential in their cytokine-secreting ability (Th0-like) at early stages after activation, producing primarily IL-2, but also synthesizing small quantities of other cytokines, including IFN-γ and IL-4. Recently activated naive and memory cells appear to be similarly susceptible to cytokines secreted by other cells (e.g., macrophages, NK cells, mast cells/basophils, or APC such as dendritic cells) and become polarized by IFN-γ and IL-12 to a Th1 phenotype, as shown in the present study, and by IL-4 to a Th2 phenotype (14, 24). However, importantly, both naive and memory CD4 cells appear to be able to regulate their own development into effectors through autocrine usage of the IL-4 and IFN-γ they produce, with the phenotype of effector populations determined by the relative amounts of IFN-γ vs IL-4 secreted.

It is evident from this study that the strength of CD4 cell stimulation had a significant effect on the level of endogenous IFN-γ secretion by CD4 cells during effector cell differentiation (Fig. 5*B*), with higher levels of IFN-γ secreted by responding T cells with optimal as opposed to suboptimal stimulation. Moreover, the ability to detect an effect of exogenous IFN-γ on Th1 development

was related to the potency of stimulation of CD4 cells (Fig. 5A). With strong stimulation, sufficient levels of endogenous IFN-γ were apparently produced to obviate the necessity of an additional source of this cytokine. However, when stimulation of CD4 cells was suboptimal, the addition of rIFN-γ resulted in a notable enhancement of Th1 effector development regardless of whether endogenous IL-4 was also neutralized. In contrast, rIL-12 greatly augmented the production of IFN-γ by CD4 cells, particularly in combination with strong stimulation (Fig. 5*B*), engendering a situation where autocrine usage of IFN-γ by CD4 cells could provide additional amplification of Th1 cell development (Fig. 6). In Ag-specific responses, such amplification might occur indirectly via induction of IL-12 production by APC. In particular, recent reports indicate that IFN-γ may up-regulate IL-12 synthesis and secretion by macrophages (45–47). A study by Flesch et al. (45) showed that IL-12 production by macrophages following infection with *Mycobacterium bovis* BCG is dependent upon IFN-γ, suggesting that in responses to some pathogens, IFN-γ may be essential for IL-12 production. In such situations, IFN-γ would play a critical, rather than a subsidiary, role in the development of Th1 cells and ultimately in the generation of a successful immune response.

In this study we also demonstrate the novel finding that IL-2 production by effectors generated from either naive or memory CD4 cells was diminished when induction occurred in the presence of either rIFN-γ or rIL-12 (Fig. 2). While IFN-γ has been previously shown to have several inhibitory activities, including antiproliferative effects on Th2 cells (48), a negative effect on the development of effectors with the capacity to secrete IL-2 has not been previously described, nor have previously published studies evaluated the effects of IL-12 on the capacity of CD4 effector cells to secrete IL-2. A role of IFN-γ in down-regulating IL-2 was indicated by the finding that neutralization of endogenous IFN-γ augmented the ability of effectors to produce IL-2, while at the same time, priming for IFN-γ secretion was reduced (Fig. 3), with levels of IL-4 and IL-5, if detectable, being unaffected (not shown). However, IL-12 was even more potent than IFN-γ in causing polarization of CD4 effectors to secretion of predominantly IFN-γ with little IL-2 (Figs. 1 and 2), presumably due at least in part to its effects on endogenous IFN-γ production (Fig. 5*B*). This was verified using CD4 cells from IFN-γ knockout mice (Fig. 6). Under the conditions of our experiments, we were unable to detect TNF-α, another cytokine often associated with Th1 cells, which could have served as an alternative indicator of Th1 development (not shown). Our results, therefore, showed that the very striking effects of IFN-γ and IL-12 on effector development, resulting in increased IFN-γ secretion, on the one hand, and decreased IL-2 production, on the other, were maximal only when both cytokines were present. Since IL-2 is initially produced by both naive and memory CD4 cells immediately following stimulation, it may be that the capacity to produce this cytokine decreases as more complete polarization to either a Th1 or a Th2 phenotype progresses. When endogenous IL-4 was not blocked in experiments with CD4 cells from IFN-γ knockout mice, effectors were generated that were Th2-like, producing both IL-4 and IL-5, confirming previous studies with these mice showing a default of CD4 effectors to a Th2 phenotype in the absence of IFN-γ (44). These data underscore an essential role of IFN-γ in inducing Th1 polarization (as defined by IFN-γ production) in the absence of IL-12.

In conclusion, the results of this study demonstrate that regulation of the development of Th1 cells by the cytokines IFN-γ and IL-12 is strikingly parallel in resting naive and memory CD4 cells and underscore that each of these cytokines can direct the development of IFN-γ-secreting effector populations independently of one another. However, both cytokines together have the most potent effects on Th1 development, resulting in effectors that secrete high levels of IFN-γ and low levels of IL-2. Because IL-12 can induce IFN-γ production by T cells, and IFN-γ can induce IL-12 production by macrophages (45–47), we envision two interdependent pathways for the development of Th1 effectors, as shown in Figure 7.

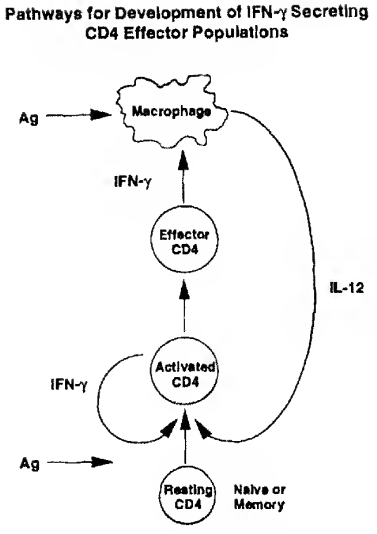

FIGURE 7. The roles of IFN-γ and IL-12 in the development of Th1 cells.

Pathogens that target the innate immune system and have the capacity to activate IL-12 production by macrophages or other APC will induce both Th1 development and IFN-γ secretion by CD4 cells in response to IL-12. Autocrine usage of IFN-γ would further amplify Th1 development under these conditions. Ag that are unable to activate macrophages directly, but have the capacity to activate CD4 cells when presented on appropriate APC would elicit IFN-γ secretion from CD4 cells themselves, with more potent stimulation leading to greater levels of IFN-γ production by T cells. Endogenous IFN-γ could then promote the development of Th1 effectors in an autocrine manner and, in addition, induce IL-12 production by macrophages, leading to amplification of Th1 development. The relative contributions or dominance of each pathway in a particular response would thus be determined by the conditions of Ag/APC stimulation. Endogenous cytokine secretion by memory CD4 cells may be of particular importance for the regulation of Th1 vs Th2 memory effector cells, since the memory response is less dependent upon costimulation and typically elicited under conditions of more limited Ag exposure where innate resistance mechanisms are less likely to come strongly into play. Since the presence of appropriate, rather than inappropriate, T cell-derived cytokines can be essential for a beneficial immune responses, these findings have important implications for vaccine strategies to potentiate the development of Th1 cells.

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